Fine Structural Demonstration of Alkaline Phosphatase Activity

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Although the histochemically demonstrating method of alkaline phosphatase activity has been developed since more than 25 years ago, the fine structural study of the localization of this enzyme activity with a high alkaline pH optimum has thus been far difficult. Nawaday, three ways are known for the fine structural demonstration of alkaline phosphatase activity.

Usually lead ions have been used as the capture reagent, but the studies of the localization of the activity of alkaline phosphatase which have a high alkaline pH optimum have been conducted below pH 8\(^3\), since at higher pH a precipitate of lead hydroxide forms. Alternatively, calcium ions have been used as capture reagent and the final product, calcium phosphate has been substituted with lead or silver ions\(^\text{1,4}\). This alternative method is usually unsatisfactory since the substitution requires two steps with increased the opportunity for diffusion of final product. On the third method, developed by Mizutani and Barrnett\(^\text{2}\) cadmium ions are used as a capture reagent for enzyme localization at a fine structural level for the range of pH 9-10.

At the present study, the fine structural localization of alkaline phosphatase activity in the rat kidney, heart and intestine was described by these three staining methods, particularly by the cadmium method, and the specificity of these staining reactions was discussed. In the kidney, alkaline phosphatase activity was localized by discrete deposition of final product (cadmium phosphate) at the brush border to the membranes covering the microvilli. The unit membranes lining apical, tubular invaginations and vesicles, which were continuous with the membranes covering microvilli, also showed activity. Final product was also deposited in relation to basal infolded membranes, but these were less active than those of the brush border. All other constituents of the cells of the proximal convoluted tubules were unreactive except for the dense bodies (lysosomes) which showed slight activity even at this pH and are known to contain acid phosphatase.

In the glomeruli the reaction product was confined to the epithelial cells, particularly to the basal portion of the plasma membrane of the podocytes which faced the unreactive fenestrated endothelial cells of glomerular capillaries. Contrary to the lack of reaction of fenestrated endothelial cells in the kidney, unfenestrated capillary endothelium of heart muscle showed activity confined only to the pinocytic vescles.

The staining patterns of absorptive cells of the small intestine which have
an alkaline phosphatase associated with the striated borders as well as with the Golgi apparatus were also described. However, the clean controls could not be obtained with the absorptive cells, since cadmium ions presumably reacted with lipid particles between, on, or in the cytoplasm.

In conclusion, none of these methods are complete for demonstration of alkaline phosphatase activity in fine structural level. Concerning the cadmium method, the variability of results should be fixed, and further studies are required.

References


Electron Microsopic Localization of Nucleoside Phosphatase Activity in Plasma Membrane of Rat Liver and Hepatomas.

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The activities of nucleoside phosphatase, especially of adenosine triphosphatase were electron cytochemically examined in the liver and ascites hepatomas of rats. For the demonstration of nucleoside phosphatase, the following procedures were employed: The prefixation using formaldehyde and glutaraldehyde were employed, Wachstein and Meisel medium and Padykula and Herman medium were used for incubation, dehydration using graded series of alcohol and embedding using Epon 812 were employed.

In the ascites hepatoma, AH 7974 and Ly 7, a variants of Yoshida sarcoma, higher adenosine triphosphatase activity was demonstrated in the plasma membrane of adjacent surface of the tumor clumps (so called hepatoma island). Also higher nucleoside triphosphatase activity was demonstrated in the junctional complex-terminial bar-of the tumor cells, the nucleoside triphosphatase of the site split adenosine triphosphate and uridine triphosphate tested, while adenosine monophosphate, uridine-5'-monophosphate and uridine-3'-monophosphate tested were not hydrolysed.

Higher nucleoside monophosphatase was demonstrated in the plasma membranes of free cell surface of the tumor cell. Adenosine-5'-monophosphate, uridine-5'-monophosphate and cytidine-5'-monophosphate were hydrolysed but 3'-monophosphates of above nucleosides and β-glycerophosphate were not hydrolysed.
It is postulated that above enzymes have an important roles in the transport mechanisms of various molecules through the terminal bar and adjacent cell membranes of the tumor cells.

It is also interesting that the difference of the activities of the nucleoside phosphatase between two different sites of the plasma membranes of island-forming hepatoma cells resembles those of hepatic parenchymal cells—bile canalicular and sinusoidal surfaces.

**Specificity Involved in the Dehydrogenase Reaction Using Tetrazolium Salts as an Electron Acceptor**

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The specificity involved in the dehydrogenase reaction demonstrated by the tetrazolium method concerns a great deal with the technical problems.

1. **Choice of the tetrazolium salt.** In terms of lipid solubility and electron density of formazans formed tetranitro-blue tetrazolium chloride (TNBT) is the best dye for the electron cytochemical demonstration of dehydrogenases such as succinic dehydrogenase at present.

2. **Choice of the fixative.** Osmium tetroxide is no more used as a fixative for the electron cytochemistry. Aldehydes are widely used in the field of electron cytochemistry. However, aldehydes such as glutaraldehyde and formaldehyde are two strong aldehydes which inhibit intramitochondrial dehydrogenase activity. Furthermore care should be taken for the fact that hydroxyadipaldehyde and crotonaldehyde can reduce TNBT to formazans in vitro at 37°C within 1 hour incubation. Glutaraldehyde and formaldehyde also reduce TNBT after overnight incubation at 37°C. None of aldehydes reduced TNBT at 0-2°C even following prolonged incubation of 3 days. This fact indicates that the aldehyde used should be washed out thoroughly after fixation and that an incubation of specimens with medium should preferably be done at near 0°C or 20°C. If an incubation has to be carried out at 37°C because of the too low enzymatic activity at near 0°C, the length of incubation should be kept minimum, not exceeding 30 minutes.

3. **The form of specimens.** Specimens can be in the form of small diced blocks or thick non-frozen sections cut by an automatic chopper. Frozen sections are not advisable because of the deteriorating effect of freezing on the fine structure.

4. **The embedding medium.** TNBT-formazans, perhaps monoformazans, are
slightly soluble in MNA, a component of a regular Epon mixture. Therefore, the use of a mixture of Epon-DDSA-DMP 30 is an absolute necessity for the prevention of the diffusion artifact of formazans formed.

5. Unfavorable formazan formation. TNBT can unfavorably be reduced by reactive SH groups in tissues. The SH groups of some amino acids such as cysteine involved in the keratinization in the epithelia of the dorsal surface of the tongue in the rodent can reduce TNBT to form formazans. The alcohol dehydrogenase which is very susceptible to an SH inhibitor may act as an endogenous dehydrogenase in the liver or kidney.

Electron Microscopic Observations on Monoamine Oxidase Activity

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Abstract

For the application of Glenner's tetrazolium method to the electron histochemical demonstration of monoamine oxidase some in vitro examination were carried out with special reference to identification and efflux of formazan and intracellular localization of the enzyme in the locus coeruleus of the rat brain was studied.

The results of examinations in vitro are summerized as follows:

1. Formazan derived from TNBT is, within ultrathin section, barely visible due to its low electron-density, while treatment with osmium tetroxide causes an increase in density of formazan.
2. Formazan coupled with non-fixed protein shows, within ultrathin section, relatively high electron density and is less affected with osmium tetroxide.
3. Considerable amount of reddish purple efflux out of the complex of formazan and glutaraldehyde fixed protein is found in the dehydrating and embedding process after osmium fixation but it is scarcely observed after glutaraldehyde fixation.

These facts may indicate that diformazan as produced in Glenner's medium is partially oxidized by osmium tetroxide and, therefore, they may support the following results obtained in tissue specimen.

Electron micrographs of the tissue including the locus coeruleus which is incubated in Glenner's medium and post-fixed with glutaraldehyde reveal that the formazan due to monoamine oxidase activity is located exclusively on mitochondria, where the deposits are almost entirely confined to the outer membrane.
On the other hand, it is also demonstrated that osmium post-fixation results in more electron opaque deposits corresponding to formazan which scarcely coincide with the site of the formazan occured in the aldehyde fixed tissue.

Histochemical and Electronmicroscopic Study on the Intracellular Localization of Phosphorylase and on the Specificity of the Histochemical Reaction for the Enzyme Activity

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Since 1954, histochemical demonstration of phosphorylase in animal tissues has been investigated by Takeuchi and his co-workers with a polysaccharide synthesizing method. A histochemical electron micrography of the enzyme activity was also tried at first by Takeuchi in Yoshida sarcoma cells in 1957. Sasaki and Takeuchi succeeded to get the electron micrograph of polysaccharide newly formed by the enzyme activity in situ by use of human muscle (J. Histochem. & Cytochem., Vol. 11, No.3, 1963).

The present work is concerned with an improved preparation and with getting a new result of phosphorylase localization. The specificity of the electron histochemical reaction was also discussed.

Material and Methods

The skeletal muscle of adult albino rats was used as the material.

In the first step, fresh frozen sections of the leg and gluteus muscle were cut with the cyostat microtome for the usual histochemical procedure. Takeuchi's polysaccharide synthesizing method and the metal precipitation method were tried in this preliminary experiment. In the synthesizing method, the Takeuchi's medium containing glucose-1-phosphate as the substrate was prepared for the incubation. In order to examine the specificity of the histochemical reaction for enzyme, various control media containing G-6-P, F-6-P, F-1-P, 6-P instead of G-1-P and the substrate free mixture were employed in the same condition. The iodine reaction was used for the histochemical demonstration of polysaccharide synthesized in situ. On some of the sections, digestion test by 3% α-amylase solution was tried. In the metal precipitation method, 0.003 M lead nitrates was added to the substrate mixture to get precipitate in situ. The lead phosphate precipitate in the tissues was stained with a diluted am-
monium sulfide solution. All of these sections were observed by the light microscope.

In the second step, the tiny pieces from the same materials were incubated in the Takeuchi mixture with 0.25M sucrose, fixed with 6.5% glutaraldehyde, and then refixed with 2% osmium tetroxide. They were dehydrated and embedded with Epon 812. Before electron microscopic use, these blocks were cut 1µ thick on ultramicrotome for light microscopic observations. The sections were mounted on slide glasses and were histochemically examined with iodine and periodic acid Schiff's reagent, or ammonium sulfide substitution. The block faces were then carefully trimmed so that the reactive part of the tissues should be saved.

In the third step, ultrathin sections of the above blocks were cut by LKB ultratome, mounted on mesh grids, and stained with lead.

The sections were examined with Hitachi HU-11A electron microscope.

**Result and Conclusion**

1. Synthesizing method
   a. Histochemical observations on the frozen sections.

   ![Fig. 1](image1.png) The electron micrograph of the muscle fibers incubated in Takeuchi medium. The sarcolemmas are extremely extended by the deposition of the polysaccharide newly synthesized in the subsarcolemmic layer. The polysaccharide is of low density and amorphous in shape at this magnification. ×4,400

   ![Fig. 2](image2.png) The polysaccharide deposition in the interfibrillar space. ×17,500
Polysaccharide newly synthesized from glucose-1-phosphate by the phosphorylase activity in the muscle fibers revealed a specific deep blue color with iodine. None of the sections incubated in the control media containing each intermediate product other than G-1-P was stained at all.

This polysaccharide was completely digested by amylase, and therefore it was removed from the muscle fibers.

b. Histochemical observations on the Epon embedded sections.

Polysaccharide synthesized in the Epon embedded sections also revealed the same blue color as in the frozen sections. Light microscopically, it was confirmed that this polysaccharide appeared to be in the sarcoplasm under sarcolemma and among myofibrils.

c. Electronmicroscopic observations.

The newly formed polysaccharide was abundantly deposited in the subsarcolemmic layer and scarcely in the interfibrillar spaces of the muscle fibers. At low magnification, it was of low density, and was speckled and mottled in shape. At higher magnification, it became obvious that the polysaccharide was localized in the sarcoplasmic matrix of areas described above, having no
direct relation to any other intracellular components such as nuclei, myofibrils and mitochondria. However, the sarcoplasmic reticulum seemed to be strongly extended and displaced by too much accumulation of the reaction product. At this magnification, the synthesized polysaccharide was show to be a fine granular appearance in a non-aggregated or monoparticulate form scattered diffusely in the areas.

On the other hand, most of all native glycogen in the muscle cells was usually exhausted from the cell matrix into the substrate solution during incubation. In rare cases, survived native glycogen was found in the muscle cells together with the newly formed polysaccharide in the same figure. In spite of closed localization, they could be easily distinguished. The native glycogen particles were of about 300A in diameter and densely stained with lead. On the other hand, the newly formed ones were of finer granular appearance and less stainable with lead.

2. Metal precipitation method

The phosphoric acid removed from G-1-P by the phosphorylase activity is captured by lead ions and deposited in the enzyme reaction sites of the cells.

a. Histochemical observations on the frozen sections.

On the histochemical and light microscopic level, the lead precipitates occurred in the same areas as observed in the iodine reaction. However, an unspecific precipitation by the lead affinity was occasionally observed in the muscle fibers and in other tissue components, particularly in the capillary walls.

b. Histochemical and electron microscopic observations on the Epon embedded sections.

In these sections, the clear cut depositions by the enzyme activity has not yet been obtained.

From the results of this experiment, it could be clarified that polysaccharide synthesized from glucose-1-phosphate is localized in the cytoplasmic matrix among various organelles and that there is a difference between native glycogen and the newly synthesized polysaccharide.